Role of mRNA Expression of Transcription Factors in Glucocorticoid Sensitivity of Peripheral Blood Mononuclear Cells and Disease State in Rheumatoid Arthritis

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ABSTRACT. ***Objective.*** To investigate the mechanisms underlying glucocorticoid (GC) resistance in rheumatoid arthritis (RA), we evaluated the suppressive effects of prednisolone (PSL) or methylprednisolone (MPSL) on the blastogenesis of peripheral blood mononuclear cells (PBMC). We also measured the expression of mRNA for transcription factors [GC receptor-α (GRα) and activator protein-1] known to be involved in the exertion of GC effects.

Methods. Twenty-six patients with RA and 17 healthy subjects were studied. IC_{50} of PSL and MPSL on the blastogenesis of PBMC stimulated with concanavalin A *in vitro* was estimated. Transcripts for GRα, c-fos, c-jun, and GAPDH genes in PBMC were quantitatively determined by real-time RT-PCR procedures.

Results. The amount of c-fos transcript in PBMC from RA patients was significantly high compared to the healthy subjects ($p = 0.001$). However, no difference was found in the amounts of mRNA of other transcription factors between the patients and healthy subjects. When PSL or MPSL IC_{50} in patients were directly correlated with patients' characteristics in RA, the duration of disease showed a significant positive correlation with PSL IC_{50} ($p = 0.035$). However, no significant association of PSL or MPSL IC_{50} with GRα, c-fos, or c-jun mRNA expression determined by RT-PCR was observed. Additionally, there were significant correlations between the amount of GRα mRNA and inflammatory indices such as erythrocyte sedimentation rate ($p < 0.001$) and C-reactive protein ($p < 0.05$) in the RA patients.

Conclusion. Chronic exposure to inflammation in RA suggests a decrease in the GC sensitivity of peripheral lymphocytes. Although c-fos and GRα transcripts in PBMC have been implicated in the pathology of RA, the amount of expression of these factors may not be critical for the development of GC insensitivity in the PBMC in RA. (J Rheumatol 2004;31:464–9)

Key Indexing Terms:
TRANSCRIPTION FACTOR ACTIVATOR PROTEIN-1 HYDROCORTISONE
GLUCOCORTICOID RECEPTORS RHEUMATOID ARTHRITIS

For many years, glucocorticoids (GC) have been the cornerstone in the treatment of autoimmune or inflammatory diseases including rheumatoid arthritis (RA), ulcerative colitis, and bronchial asthma. There are, however, a proportion of patients with RA who do not benefit from GC therapy. The individual lymphocyte responsiveness to GC determined by the mitogen proliferation assay has been reported to be useful to identify GC-insensitive patients before they undergo GC treatment[1-5].

It has been reported that the effect of GC is mainly mediated by their binding to GC receptor (GRα), a well investigated receptor isoform. GRα is categorized as a transcription factor in that it interacts with other transcription factors (trans-repression) and also regulates gene expression by binding the GC responsive element (GRE) (trans-activation)[6]. After binding with GC, GRα homodimerizes and binds to GRE located in the promoter region of genes encoding antiinflammatory mediators, and consequently trans-activates such gene expression. Meanwhile, GC-GRα complex interacts directly with other transcription factors such as activator protein-1 (AP-1) and nuclear factor-κB (NF-κB), and inhibits the induction of proinflammatory cytokine gene expression.

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AP-1, a heterodimer of Fos and Jun oncoproteins, binds to tetradecanoyl phorbol acetate-response element, which has been found in the promoters of genes encoding collagenase, matrix metalloproteinases, and interleukin 1 that are essential in the pathology in RA[7]. Indeed, it has been reported that c-fos expression and AP-1 DNA binding are constitutively upregulated in RA synovium[8].

GC-GRα complex interacts with AP-1, and these transcription factors attenuate their effects mutually. Excessive AP-1 results in the deprivation of effective GRα complex, decreasing the amount of GRα that exerts the effect of transactivation. It has been observed that the expression of c-fos and DNA binding of AP-1, but not NF-κB and cyclic AMP response element binding protein, is increased in GC-resistant asthmatic patients compared to GC-sensitive ones, suggesting that AP-1 also contributes to acquiring desensitization to GC[9,10].

Although the role of transcription factors such as AP-1 in the pathogenesis of RA has been described recently[8,11], to date the relationship between the expression amount or function of AP-1 and GC efficacy in RA has not been investigated. Since inflammatory cytokines and related molecules that play major roles in the pathogenesis of each autoimmune disease are different, the mechanism(s) for GC resistance in RA should be investigated. To determine the association of AP-1 and GRα with the efficacy of GC in RA, we investigated the relationship between the expression of mRNA for AP-1 or GRα and lymphocyte sensitivity to GC using a mitogen proliferation assay.

MATERIALS AND METHODS

Twenty-six Japanese patients diagnosed with RA and 17 healthy controls were studied (male/female: 5/21 for patients and 2/15 for controls). The mean age was 55.8 ± 13.6 years (range 28–82) in patients and 44.4 ± 9.8 years (range 31–66) in controls. No patient had complications of immunological disorders other than RA. At the time of blood sampling no patient had experience of GC administration, or alternatively had had a washout period of at least 3 months from the last GC treatment. Patients being administered methotrexate were excluded. Erythocyte sedimentation rate (ESR), serum C-reactive protein (CRP) concentration, rheumatoid factor (RF), and the white blood cell (WBC) count at the time of blood sampling were also measured.

Isolation of PBMC. With patients' informed consent, venous blood was taken between 1:00 PM and 3:00 PM. The heparinized blood was loaded on Ficoll-Hypaque and centrifuged. PBMC, including lymphocytes, were separated as described[12,13].

PBMC culture and evaluation of drug effects. Two hundred microliters of cell suspension were placed into each of the 96 flat-bottom wells of a microtiter plate. Concanavalin A (ConA) as a mitogen was added to each well to a final concentration of 5.0 µg/ml. Subsequently, 4 µl of ethanol solution containing prednisolone (PSL) or methylprednisolone (MPSL) were added to yield final concentrations of 0.1, 1.0, 10, 100, 1000, and 10,000 ng/ml (corresponding to 2.8×10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M PSL and 2.7×10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, and 10^{-5} M MPSL, respectively). Four microliters of ethanol were then added to the control wells. The plate was incubated 96 h in 5% CO_2/air at 37°C in a humidified chamber. The cells were pulsed with 18.5 kBq/well of [^{3}H] thymidine for the last 16 h of incubation and then collected on glass-fiber filter paper using a multiharvester device and dried. The radioactivity retained on the filter was processed for liquid scintillation counting. The mean of the counts for duplicates of each sample was determined, and the agent concentration that would result in 50% lymphocyte-mitosis inhibition (IC_{50}) was determined from the dose response curves.

Extraction of RNA and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). PolyA(+)RNA was isolated from patients' PBMC using a QuickPrep polyA(+)RNA extraction kit (Amersham Pharmacia Biotech, Buckinghamshire, UK) according to the manufacturer's instructions. cDNA was constructed by RT in 50 µl buffer containing 1× TaqMan buffer, 5.5 mM $MgCl_2$, 500 µM of each dNTP, 2.5 µM of random hexamer, 0.4 U/µl of RNase inhibitor, and 1.25 U/µl of MultiScribe reverse transcriptase. RT was performed with a stepwise reaction at 25°C for 10 min, 48°C for 30 min, and 95°C for 5 min. For the subsequent PCR procedure, 2.5 µl cDNA mixture was used. To perform the negative control experiment, reverse transcriptase was omitted for each sample.

The PCR was performed using real-time SYBR green technology and analyzed by an ABI 7700 sequence detector (Applied Biosystems, Foster City, CA, USA). Primer pairs for GRα, c-fos, c-jun, and GAPDH were designed to amplify 161 bp, 86 bp, 111 bp and 226 bp products, respectively. Primer sequences were as follows: GRα, 5-CAAAGAGCTAGGAAAAGCCATTG-3 and 5-CAATACTCATGGTCTTATCCAAAAATGT-3; c-fos, 5-AGGAGAATCCGAAGGGAAAGG-3 and 5-TCCGCTTGGAGTGTATCAGTCA-3; c-jun, 5-CCTTTCCTGCGTCTTAGGCTT-3 and 5-AGTTCAACAACCGGTGCGAG-3; GAPDH, 5-GAAGGTGAAGGTCGGAGTC-3 and 5-GAAGATGGTGATGGGATTTC-3. To compare the expression level of mRNA among the subjects, the ratio of GRα, c-fos, or c-jun transcript to GAPDH transcript was calculated. Each sample was analyzed in duplicate and all PCR products were subjected to electrophoresis using 2% agarose gel to ensure that no unspecific amplicon was obtained. To further confirm the sequences, the PCR products were applied to sequencing analysis by an automated DNA sequencer (ABI 310; Applied Biosystems).

Measurement of plasma cortisol concentrations. Plasma cortisol measurement was performed by high performance liquid chromatography (HPLC) with minor modification of the procedure[14]. To analyze plasma samples, we added 500 µl of 0.2 mol/l acetate buffer (pH 3.85) to 1 ml of plasma before extraction. MPSL (Sigma, Tokyo, Japan) as an internal standard was added to all samples before extraction. The plasma samples were centrifuged and GC were extracted with Sep-Pak Vac 3 ml C18 cartridges (Waters, Tokyo, Japan) preactivated with 3 ml of methanol followed by 3 ml water. After the cartridges were positioned on a 10-port vacuum elution manifold (Varian, Harbor City, CA, USA), we washed them under reduced pressure with 3 ml of each in the following order: acetone:water (25:75 by volume), water, and hexane. GC were subsequently eluted with 3 ml diethyl ether into glass tubes. The effluents were dried under nitrogen and reconstituted in 200 µl of the mobile phase; 20 µl of each reconstituted sample were injected into the HPLC device (Shimazu, Kyoto, Japan).

Data analysis. An unpaired T test or Mann-Whitney U test was used, when appropriate, to compare data between the 2 groups. Age matching was performed for comparison of certain indices between RA patients and controls. Pearson's correlation coefficient test was applied to investigate the correlation between the 2 factors. When IC_{50} were applied to the Pearson test, these values were logarithmically transformed. P values less than 0.05 were regarded as statistically significant. All analyses were performed using GraphPad Prism 3.0 software (GraphPad Software, San Diego, CA, USA).

RESULTS

Comparison of GRα, c-fos, and c-jun mRNA expression in PBMC. The amount of GRα, c-fos, and c-jun mRNA in patient and control PBMC was compared. The amount of

GRα expression ranged from 0.013 to 0.686/GAPDH mRNA in the patients (n = 18) and from 0.091 to 0.277/GAPDH mRNA in controls (n = 17) after age matching. The amount of c-fos mRNA in the PBMC ranged from 0.197 to 16.60/GAPDH mRNA in patients (n = 19) and from 0.03 to 9.75/GAPDH mRNA in controls (n = 17). The amount of c-fos mRNA in PBMC from patients was significantly higher than that from controls (p = 0.001, Mann-Whitney U test; Figure 1). The amount of c-jun mRNA in the PBMC ranged from 0.004 to 0.240/GAPDH mRNA in the patients (n = 19) compared to 0.012 to 0.140/GAPDH mRNA in the controls (n = 17). There was no significant difference in the amount of GRα or c-jun mRNA expression between RA patients and controls (data not shown).

Association of GC sensitivity of RA PBMC with clinical profiles or mRNA expression of GRα, c-fos, and c-jun. In each RA patient, PSL and MPSL concentrations that would give 50% lymphocyte-mitosis inhibition (IC_{50}) *in vitro* were determined from the dose response curves. IC_{50} for PSL and MPSL ranged from 2.7 to 1000 ng/ml and 0.1–1318 ng/ml, respectively, and thus there were large individual differences of IC_{50} for both PSL and MPSL in the RA patients. The IC_{50} for PSL correlated significantly with the IC_{50} for MPSL (r = 0.85, n = 25, p < 0.0001; Figure 2). When IC_{50} data for PSL were correlated directly with patients' RA characteristics, disease duration showed a significant correlation with PSL IC_{50} (p = 0.035; Table 1). Other disease variables such as ESR, CRP, RF, and WBC counts did not show significant associations with PSL IC_{50} in RA patients. Additionally, MPSL IC_{50} in patients were found to be significantly associated with WBC counts (p = 0.004), while MPSL IC_{50} were not found to be significantly associated

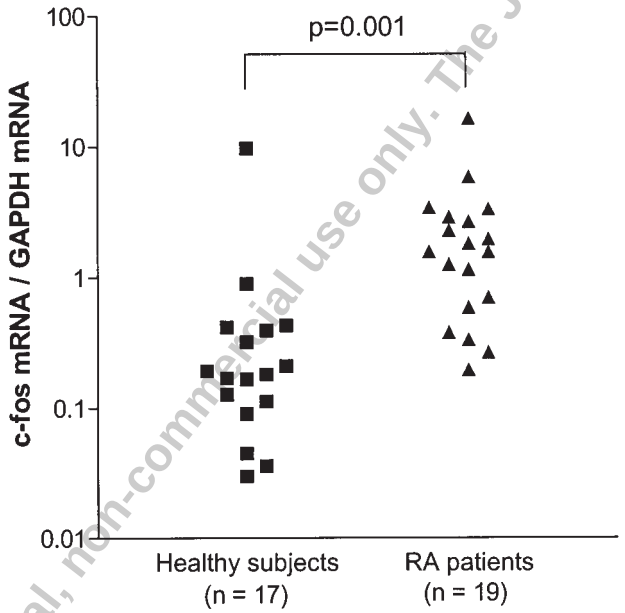

Figure 1. Comparison of relative amount of c-fos mRNA expression in PBMC between patients with RA and age matched healthy controls.

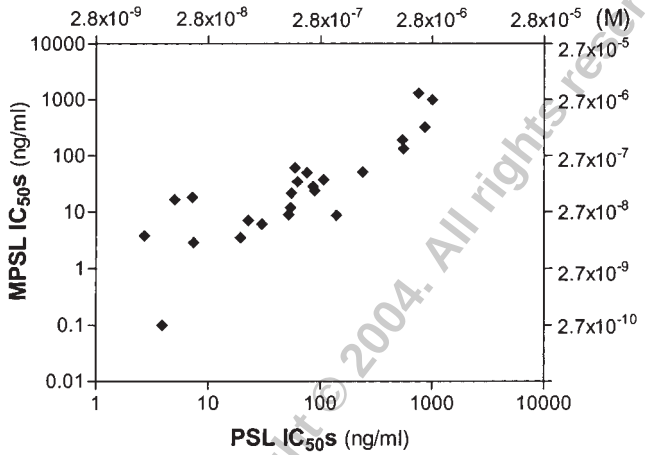

Figure 2. Correlation between IC_{50} for PSL and MPSL in 25 patients with RA (p < 0.0001). IC_{50} were determined from dose response curves as a GC concentration that required 50% inhibition of PBMC proliferation stimulated by concanavalin A.

with age, ESR, CRP, RF, or duration of disease. To further investigate the underlying individual differences for the lymphocyte sensitivity to GC, PSL or MPSL IC_{50} were directly correlated with the amounts of GRα, c-fos, or c-jun transcripts. However, no significant association was observed between GRα, c-fos, or c-jun mRNA expression and GC sensitivities in PBMC (Table 1).

Relationship between GRα, c-fos, or c-jun expression and patients' characteristics. We investigated the relationship between the amounts of GRα, c-fos, or c-jun transcripts expressed in the PBMC and clinical profiles of the patients with RA. We observed a significant negative correlation between the amount of GRα mRNA expression in the PBMC and ESR in these patients (p < 0.001; Figure 3A). Similarly, CRP was also negatively correlated with the amount of GRα mRNA expression (p < 0.05; Figure 3B). We also investigated the contribution of plasma cortisol concentrations to GRα mRNA expression. Plasma cortisol concentrations deviated from 12.7 to 110.2 ng/ml (mean ± SD 60.7 ± 26.4 ng/ml) in these patients. However, plasma cortisol concentrations did not correlate significantly with IC_{50} for GC (Table 1), GRα mRNA expression, or disease activity indices (data not shown).

DISCUSSION

It has been shown that lymphocyte sensitivity to GC *in vitro* determined by the inhibition of ConA-stimulated peripheral blood lymphocytes correlates with the clinical outcome of GC treatment in RA[5]. However, the molecular mechanisms underlying the individual differences of lymphocyte sensitivity to GC in RA have yet to be clarified. To investigate the factors that contribute to the development of GC insensitivity, we directly correlated PSL or MPSL IC_{50} with the expression of mRNA for transcription factors, clinical characteristics, and laboratory data. We found that there was a

Table 1. Pearson correlation analysis between IC_{50} of PSL or MPSL against PBMC blastogenesis and RA patient characteristics.

	Age, yrs	ESR, mm/h	CRP, mg/dl	RF, IU/ml	WBC (x 10^3/mm)	Duration of Disease, yrs	Relative amount (/GAPDH mRNA) of mRNA for: GRα	c-fos	c-jun	Plasma Cortisol, ng/ml
PSL IC_{50}										
N	26	25	26	25	26	26	24	25	24	25
Pearson r	–0.083	0.094	0.257	0.033	0.312	0.414	–0.037	-0.013	0.096	0.195
p	0.689	0.656	0.205	0.875	0.121	0.035	0.863	0.951	0.656	0.351
MPSL IC_{50}										
N	25	24	25	24	25	25	23	24	23	25
Pearson r	–0.012	–0.063	0.225	0.042	0.550	0.363	0.025	0.054	0.004	0.093
p	0.954	0.771	0.281	0.846	0.004	0.075	0.912	0.804	0.987	0.657

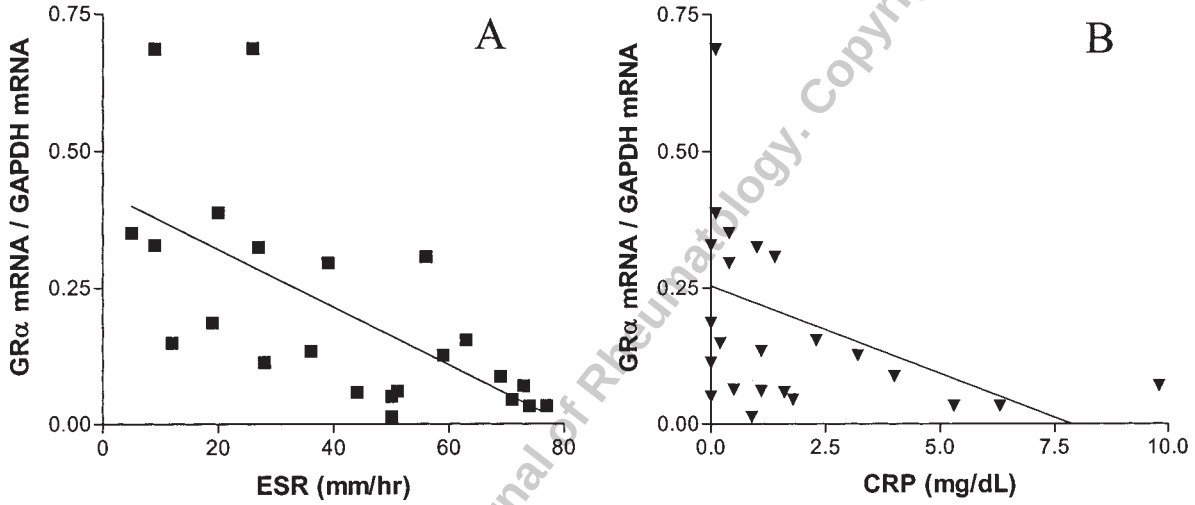

Figure 3. Significant negative correlations between relative amount of GRα mRNA expression and ESR (A) ($p < 0.001$) or CRP (B) ($p < 0.05$) in PBMC from patients with RA.

significant correlation between PSL sensitivity of PBMC and disease duration. PBMC sensitivity to MPSL also tended to be associated with disease duration, although the relationship was not statistically significant ($p = 0.075$). Reports have shown that there is no difference in disease activity or disease-related clinical indices between GC insensitive/resistant patients and GC sensitive patients with RA or inflammatory bowel diseases, suggesting that GC sensitivity is independent of disease activities[5,15,16]. Although we did not find an association of GC sensitivity of PBMC with disease activity of RA, our observations suggest that chronic inflammation in RA may affect the GC sensitivity of peripheral lymphocytes. Thus, the data also raised the possibility that early intervention with GC treatment is more effective in the control of the disease.

Additionally, we observed significant positive correlation of MPSL IC_{50} with WBC counts in RA patients, whereas there was no association of PSL IC_{50} with WBC counts. Although the relationship in 25 RA patients was statistically significant, this might not be applicable to the GC insensitive/resistant subjects, because the correlation was not significant without one patient whose MPSL IC_{50} and WBC counts were extremely small. Nevertheless, further research will validate this association. To investigate the association of molecular characteristics with GC sensitivity of PBMC, we performed real-time RT-PCR to measure the spontaneous expression of mRNA for GRα, c-fos, and c-jun, which have been reported to be implicated in GC effects. However, no significant relationship of the expression levels of these transcripts with GC sensitivities of PBMC was observed in the patients with RA.

Although a previous report has described that the amount of GR determined by receptor binding assay in PBMC of patients with lupus nephritis correlates with the clinical outcome of GC treatment[17], this is not applicable to all rheumatic diseases[18]. It is true that the qualitative or quantitative characteristics of GR do play a role in GC efficacy; recent studies have shown that post-receptor mechanisms

are also involved. Lane, *et al* reported that c-fos in PBMC is induced in GC-resistant asthma under basal conditions as well as the phorbol 12-myristate 13-acetate-stimulated condition as compared to GC-sensitive asthma[10]. Increased AP-1 interacts with GRα and inhibits the DNA binding of GRα, which also attenuates the antiinflammatory effect of GC[6]. Additionally, we have recently shown that the amount of c-fos mRNA expressed in PBMC correlates with GC sensitivity *in vitro* in asthmatic patients[19]. While we measured the amount of mRNA for c-fos in PBMC without stimulation in the current study, the change of expression levels of these transcripts in a stimulated condition *in vitro* might provide some clue to understanding the individual differences of GC sensitivity in RA.

One of the other candidates that may contribute to GC insensitiveness in RA is GRß, an alternative splicing isoform of GRα. GRß is unable to bind GC and is reported to be a dominant negative inhibitor of GC-GRα complex. There have been some controversial observations in terms of the dominant negative effect of GRß[20-22], although it has been reported that GRß expression in PBMC and/or topical tissue is associated with clinical GC resistance in asthma, chronic lymphocytic leukemia, and ulcerative colitis[16,23,24]. We also performed a qualitative assay for GRß mRNA expression; however, we found no tendency that GRß was expressed dominantly in GC-insensitive PBMC in this experiment.

Asahara, *et al* have shown that AP-1 DNA binding and c-fos mRNA expression are upregulated in RA synovial tissues and infiltrating lymphocytes[8]. They also described AP-1 DNA binding activity quantified by densitometry correlates with CRP concentrations. We found that c-fos mRNA expression in PBMC in RA patients was significantly higher than that of the healthy controls, suggesting that AP-1 at the circular level is also involved in the pathophysiology of RA. However, there was no significant correlation in the amount of c-fos transcript in PBMC with RA disease activity indices (ESR or CRP).

Our results also showed a significant negative correlation of GRα mRNA expression in PBMC with ESR or CRP levels. This observation prompted us to examine plasma cortisol concentrations at the time of blood sampling for the measurement of transcripts, as we thought this downregulation of GRα mRNA might result from a hypothalamus-pituitary-adrenal (HPA) axis anomaly influenced by the inflammatory conditions of RA. However, there was no correlation between plasma cortisol concentration and GRα expression or each inflammatory index.

Several studies have investigated the GR numbers and affinities determined by using receptor binding assay in PBMC from patients with rheumatic diseases. Schlaghecke, et al have shown that the number of GR in PBMC from RA patients is significantly lower than in healthy subjects[25]. On the other hand, Gladman, *et al* have shown that the number of GR in patients with systemic lupus erythematosus (SLE) is significantly higher compared to healthy subjects[26]. Thus, although reports regarding the amount of GR in rheumatic diseases are contradictory, a dose-dependent GR downregulation has been observed in subjects with several rheumatic diseases including RA, SLE, and vasculitis[27]. When it comes to the association of the number of GR with disease activity, it seems to be generally accepted that the number of GR does not correlate with inflammatory indices in RA and SLE[25,26,28]. We have for the first time quantified the mRNA expression for GR in RA patients and demonstrated its negative correlation with the disease activity of RA. The inconsistency of our results in contrast to previous studies might be due to the different susceptibilities to change in the amounts of expression between the levels of mRNA and of proteins. It has been reported that when exogenous GC is administered, it takes 2 to 3 hours for the amount of GR mRNA to be affected, while it takes 12 to 24 hours in the case of proteins[27]. Thus, one may speculate that the amount of mRNA is more responsive to environmental change including the circadian rhythm of cortisol than protein, and this might be one of the reasons for the discrepant observations.

Previous reports also conflict in terms of the correlation of cortisol concentration with RA disease activity expressed as ESR. Boss, *et al* have shown there is a significant positive correlation between ESR and cortisol concentration[29], while Schlaghecke, *et al* could not observe any correlation between baseline cortisol concentration and disease activity or GR density determined by receptor binding assay[25]. Our report is consistent with the findings of the latter study. It has been suggested that the circadian secretion pattern of cortisol concentration is completely lost in highly active RA[25]. It has also been observed that the salivary cortisol concentration in RA patients with ESR > 20 mm/h is persistently high in the afternoon, whereas the salivary cortisol concentration of RA patients with low ESR and healthy controls is normally suppressed in the afternoon[30]. Thus, the circadian rhythm of cortisol concentration may be a key for examining the association of the inflammation with cortisol concentration.

In summary, we examined the implication of overexpression of transcripts for AP-1 and GRα in the pathophysiology and GC sensitivity of patients with RA. The data suggest that (1) increased c-fos mRNA expression in PBMC is related to the pathogenesis of RA; (2) chronic inflammation in RA is related to a decrease in GC sensitivity *in vitro*; and (3) changes in amounts of GRα, c-fos, and c-jun mRNA may not be related to GC sensitivity in the PBMC of patients with RA. While we found no correlation between the amounts of AP-1 or GRα mRNA and GC sensitivity in PBMC of patients with RA, the data implied that the molecular mechanism underlying GC insensitiveness in RA should be distinguished from that of asthma.

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